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G-proteins in differentiation of epiphyseal chondrocytes

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Abstract

Here we reviewed regulation of differentiation of the growth plate chondrocytes by G-proteins. In connection with this we summarized the current knowledge regarding each family of G-protein alpha subunit, specifically, $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_{i/o}$. We discussed different mechanisms involved in chondrocyte differentiation downstream of G-proteins and different G-protein coupled receptors (GPCRs) activating G-proteins in the epiphyseal chondrocytes. We concluded that among all G-proteins and GPCRs expressed by chondrocytes, $G\alpha_s$ has the most important role and prevents premature chondrocyte differentiation. Receptor for parathyroid hormone (PTHR1) appears to be the major activator of $G\alpha_s$ in chondrocytes and ablation of either one leads to accelerated chondrocyte differentiation, premature fusion of the postnatal growth plate and ultimately short stature.

The growth plate

The majority of bones in the skeleton are formed via the process of endochondral bone formation. During this process a cartilage template is first formed and then replaced by true bone tissue. Formation of skeletal elements starts with condensation of mesenchymal cells with their subsequent differentiation into chondrocytes (Hall and Miyake 1995). Chondrocytes proliferate thereby increasing the size of the primary skeletal element. Cells in the center of the element stop proliferating, change their genetic program, and enlarge (hypertrophic differentiation), further facilitating growth of the skeletal element. Cartilage elements are avascular and reach a substantial size triggering hypoxia in the middle of the skeletal element. Hypertrophic chondrocytes secrete vascular endothelial growth factor (VEGF) that attracts blood vessels (Zelzer, et al. 2004). Invasion by blood vessels is accompanied by closely associated pre-osteoblasts from surrounding perichondrium (a thin layer of flat undifferentiated cells surrounding every cartilage element) into the center of the element (Maes, et al. 2010). Thus, true bone tissue starts to be formed by osteoblasts in the center of cartilage anlagen, creating the primary ossification center. In the case of long bones, the secondary ossification centers are subsequently formed in a similar manner at the ends of the growing cartilage template. The secondary ossification center develops into the epiphysis and primary ossification center develops into the metaphysis and diaphysis. The

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cartilage remaining between the epiphysis and metaphysis forms the growth plate, a disc with spatially organized chondrocytes. The growth plate can be morphologically divided into three zones: a resting zone with round stem-like chondrocytes located toward the epiphysis, a flat cell zone with proliferating chondrocytes in the middle, and a hypertrophic zone containing differentiated enlarged chondrocytes located toward the metaphysis (Figure 1). Chondrocytes from the resting zone are recruited into the flat zone, where they go through several cycles of proliferation, thereby substantially increasing in number and thereafter further differentiate into hypertrophic chondrocytes. As soon as cells cease proliferation and become pre-hypertrophic (changing from flat to round appearance) they start expressing the genes for indian hedgehog (Ihh) and the receptor for parathyroid hormone (PTHR1). Ihh is a secreted molecule that diffuses throughout the cartilage and stimulates production of parathyroid hormone related peptide (PTHrP) by resting cells at the top of the growth plate. PTHrP, in turn, diffuses back to pre-hypertrophic cells, binds to the PTHR1 and inhibits chondrocyte differentiation. This inhibition increases the distance between the resting zone and the pre-hypertrophic chondrocytes and decreases levels of Ihh, which reach cells of the resting zone. This expansion of the growth plate and decrease in Ihh, in turn, leads to a decrease of PTHrP expression and a decrease of the distance between round and pre-hypertrophic cells. Thus, together Ihh and PTHrP form a feedback loop, which controls the height of the growth plate (reviewed by (Kronenberg 2003)). Subsequent chondrocyte differentiation is associated with dramatic cell enlargement (hypertrophy); this cellular enlargement accounts for 59% of bone lengthening (up to 73% with associated matrix (Wilsman, et al. 1996)). Eventually hypertrophic chondrocytes die, allowing new bone to be formed on the cartilage template. The zone of cartilage to bone transition is called the chondro-osseous junction and the entire process is called endochondral bone formation.

Thus, the rate of bone growth depends on the following steps: recruitment of stem-like quiescent cells into flat zone, chondrocyte proliferation, differentiation from flat to hypertrophic chondrocytes, chondrocyte enlargement and, finally, replacement of hypertrophic chondrocytes by bone tissue. The rates of all these processes are tightly balanced; deregulation in the rate of any of them will lead to growth abnormalities, usually leading to short stature.

G-proteins

G-protein coupled receptors (GPCR), a large family of seven-transmembrane domain receptors, mediate their signaling via heterotrimeric G-protein complexes, which consists of alpha, beta and gamma subunits. Sixteen genes encode Gα subunits, five encode Gβ, and 12 genes encode Gγ subunits. When inactive, guanosine-diphosphate (GDP) is bound to the alpha subunit stabilizing the complex between alpha, beta and gamma. Upon activation of the GPCR, the receptor functions as a nucleotide exchanger: GDP is replaced by the more abundant guanosine-triphosphate (GTP). This leads to dissociation of Gα-GTP from Gβγ and allows Gα-GTP to activate downstream effectors. The Gβγ dimer also may mediate signaling (reviewed in (Gautam, et al. 1998; Oldham and Hamm 2008)), but the role of this signaling pathway in chondrocytes is largely unknown. The Gα-GTP complex remains active until GTP is hydrolyzed. $G\alpha_s$ has an intrinsic hydrolytic activity (GTPase), which is enhanced by Regulators of G protein signaling (or RGS). There are about 30 different

RGS's and their role in bone is comprehensively discussed in a recent review (Keinan, et al. 2014).

Based on similarity of their α-subunits sixteen members of the Gα subunit family are divided into four major classes: $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_{i/o}$ (Freissmuth, et al. 1989; Neves, et al. 2002; Simon, et al. 1991).

The Stimulatory alpha subunit ($G\alpha_s$) family consists of three members: $G\alpha_s$ small and large splice isoforms of the same GNAS1 gene and $G\alpha_{olf}$. Active $G\alpha_s$ activates adenylate cyclase, an enzyme that catalyzes the synthesis of cAMP from ATP within cells. GPCRs are activated by many types of ligands, including glycoproteins, many peptide hormones, catecholamines and neurotransmitters; all such types of ligands can activate $G\alpha_s$ to raise intracellular cAMP in their target cells. cAMP works as a second messenger activating several downstream targets including protein kinase A (PKA, also known as the cyclic AMP-dependent protein kinase or A kinase), cAMP-regulated guanine nucleotide exchange factors 1 and 2 (Epac1 and Epac2) and cyclic nucleotide-gated ion channels (Freissmuth et al. 1989; Kaupp and Seifert 2002; Neves et al. 2002; Simon et al. 1991).

The $G\alpha_{q/11}$ family includes four members, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15}$. Activation of $G\alpha_{q/11}$ leads to activation of PLCβ, which cleaves phosphatidylinositol-4,5- bisphosphate (PIP2; also known as PtdIns(4,5)P_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), leading to calcium mobilization and protein kinase C (PKC) activation. In turn, PKC can activate guanine nucleotide exchange factors of the Rho family (Rho GEFs) and MAPK cascades. In addition, $G\alpha_q$ can activate AKT kinase, which modulates mTOR and NF-kB signaling pathways (Freissmuth et al. 1989; Neves et al. 2002; Simon et al. 1991).

The $G\alpha_{12/13}$ family consists of two members, $G\alpha_{12}$ and $G\alpha_{13}$. Activation of these proteins induces Rho GEFs, which initiate Rho-dependent signaling through Rho-associated coiled-coil containing protein kinase (ROCK) and p38 MAPK (Freissmuth et al. 1989; Neves et al. 2002; Simon et al. 1991).

The $G\alpha_{i/o}$ family includes 9 members: $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{0a}$ and $G\alpha_{0b}$ (splice isoforms), $G\alpha_z$, $G\alpha_{t1}$, $G\alpha_{t2}$, and $G\alpha_g$. Signaling events downstream of this family include inhibition of cAMP and calcium channels and activation of K+ channels, SRC, phosphoinositide 3-kinase (PI3K), RAC–MEK–ERK and CDC42–PAK, and Rho-mediated signals..

Below we discuss the role of each of these signaling pathways in chondrocyte differentiation in the growth plate. The data characterizing expression of different G-proteins in the growth plate are quite limited and here we assume that expression is ubiquitous, but easiest to see in prehypertrophic and hypertrophic chondrocytes as it is seen for $G\alpha_s$, $XL\alpha_s$, $G\alpha_{13}$ (Chagin, et al. 2014).

$G\alpha_s$ and chondrocyte differentiation

It appears that $G\alpha_s$ is a major participant in chondrocyte differentiation, and it inhibits the differentiation process. In humans, mutation of the $G\alpha_s$-encoding gene, GNAS1, leads to Albright Hereditary Osteodystrophy (AHO), which is associated with growth abnormalities,

short stature, brachydactyly, and premature fusion of the growth plates (Steinbach, et al. 1965). The mechanism underlying these abnormalities has been explored using genetic mouse models. Targeted ablation of $G\alpha_s$ in fetal (Sakamoto, et al. 2005) or postnatal (Chagin et al. 2014) chondrocytes leads to dramatic acceleration of chondrocyte differentiation and cessation of longitudinal bone growth. Chimeric mice with cells deficient in the GNAS1 gene demonstrate ectopically differentiated chondrocytes throughout the growth plate (Bastepe, et al. 2004), further supporting the role of $G\alpha_s$ in prevention of chondrocyte differentiation. There is a splice isoform of $G\alpha_s$ (namely extra-large stimulatory alpha subunit (XLαs)), which has an alternative exon 1, with the rest of the protein identical to $G\alpha_s$ and capable of activating the downstream cyclic AMP (cAMP) signaling pathway (Klemke, et al. 2000; Pasolli, et al. 2000). Thus, this splice isoform may partially compensate for the lack of $G\alpha_s$. Despite being expressed by hypertrophic chondrocytes, the functional role of XLαs seems to be negligible, since simultaneous ablation of XLas and $G\alpha_s$ appears phenotypically indistinguishable from $G\alpha_s$ ablation alone (Chagin et al. 2014).

Multiple *in vitro* experiments with administration of forskolin (adenylate cyclase activator) or 8-cAMP (long-lasting analog of cAMP) confirm the inhibitory effect of this pathway on chondrocyte differentiation (Jikko, et al. 1996). Interestingly, activation of cAMP pathway by dbcAMP (N^6,2'-*O*-dibutyryl cAMP) in differentiated cultured rabbit chondrocytes inhibits ALPase activity and collagen type X expression, markers of hypertrophic chondrocytes (Jikko et al. 1996) and treatment of metatarsal bones with forskolin inhibits expression of type X collagen by hypertrophic chondrocytes (Chagin, unpublished observation). These findings suggest high plasticity of the hypertrophic differentiation stage. Altogether these observations demonstrate that chondrocyte hypertrophy is tightly controlled by the $G\alpha_s$ signaling pathway.

Canonical signaling downstream of $G\alpha_s$ includes activation of adenylate cyclase, followed by cAMP accumulation and subsequent activation of PKA, which in turn lead to phosphorylation and activation of cAMP response element binding protein (CREB) transcription factor (Neves et al. 2002). However, in addition to PKA, high levels of cAMP activate Epac1 and Epac2 (cAMP-regulated guanine nucleotide exchange factors 1 and 2), whereas PKA also has multiple additional targets, including Sox9 transcription factor (Huang, et al. 2000).

The highest levels of both $G\alpha_s$ mRNA and active PKA are observed in pre-hypertrophic and early hypertrophic chondrocytes (Chagin et al. 2014). Phosphorylation of CREB at Serine 133, a PKA-phosphorylation site, is also observed in pre-hypertrophic chondrocytes (Long, et al. 2001). However, expression of dominant negative CREB in chondrocytes delays cell differentiation (Long et al. 2001) in contrast to the accelerated differentiation upon genetic ablation of $G\alpha_s$. Furthermore, the levels of phospho-CREB were not changed upon ablation of the PTHR1 (Long et al. 2001), a major activator of $G\alpha_s$ signaling in chondrocytes. Thus, the $G\alpha_s$-dependent inhibition of chondrocyte differentiation is unlikely to be mediated by CREB.

To date there are no reports of chondrocyte-specific PKA ablation, but global inactivation of the ubiquitously expressed C-alpha catalytic subunit of PKA results in early post-natal

lethality in 73% of animals (Skalhegg, et al. 2002). The remaining 27% survive till weaning and showed marked growth retardation, which, according to the authors, is associated with low serum IGF1 levels (Skalhegg et al. 2002). Unfortunately the growth plate morphology was not presented for these animals and it is difficult to determine the exact mechanisms of growth retardation in this global PKA knockout model. In these mice only one of the two PKA catalytic subunits was removed. Thus, the milder growth plate phenotype of these mice as compared to the chondrocyte-specific ablation of $G\alpha_s$ (lethal) is likely due to activity of the remaining catalytic subunit Cβ.

An exciting downstream mechanism of $G\alpha_s$- and PKA-dependent regulation of chondrocyte differentiation was recently proposed by Prof. Lassar AB (Harvard Medical School). *In vitro* experiments from his group suggest that activation of PKA stimulates serine/threonine protein phosphatase 2A (PP2A), which in turn removes phosphate from serine 246 of class IIa histone deacetylase HDAC4. This decreases binding of HDAC4 to 14-3-3 protein and allows translocation of HDAC4 to the nucleus, where it represses transcription activated by Mef2C (Kozhemyakina, et al. 2009). The importance of both HDAC4 and Mef2C for chondrocytes differentiation was convincingly demonstrated *in vivo* (Arnold, et al. 2007; Vega, et al. 2004). This mechanism outlines a plausible downstream pathway toward $G\alpha_s$- and PKA-mediated inhibition of chondrocyte differentiation and has now been confirmed *in vivo* (Nishimori, et al. 2012).

Other targets downstream of $G\alpha_s$, which are likely involved in $G\alpha_s$ -regulated chondrocyte differentiation, include the suppression of the cyclin-cdk inhibitor p57; the PKA-dependent phosphorylation and activation of the transcription factor Sox9; and the suppression of the transcription of Runx2, probably as a consequence of suppression of Mef2C action (reviewed by (Kronenberg 2006)).

The large family of G-protein coupled receptors (GPCRs) is responsible for activating $G\alpha_s$. There are several GPCRs expressed by chondrocytes that can activate $G\alpha_s$. These include PTHR1 (Lee, et al. 1993), receptors for prostaglandins (Clark, et al. 2005; de Brum-Fernandes, et al. 1996), membrane estrogen receptor GPER (Chagin and Savendahl 2007), RDC1 receptor (Jones, et al. 2006), adenosine (nucleotide P2Y) receptors (Hoebertz, et al. 2000; Kaplan, et al. 1996), β-adrenergic receptors (Lai and Mitchell 2008), histamine H2 receptors (Fukuda, et al. 1993) and probably others. Despite the fact that several of these receptors are known to signal through $G\alpha_s$, PTHR1 seems to be the predominant activator of $G\alpha_s$ in the growth plate. Indeed, ablation of PTHR1 either recapitulates the $G\alpha_s$ cKO phenotype or results in a more profound effect. Specifically, ablation of either $G\alpha_s$ or PTHR1 in chondrocytes during development (utilizing collagen 2-driven Cre) results in almost identical phenotypes (compare (Kobayashi, et al. 2002) and (Sakamoto et al. 2005)). Postnatal ablation of PTHR1 in chondrocytes has a more profound phenotype than ablation of $G\alpha_s$ (Chagin et al. 2014; Hirai, et al. 2011): ablation of PTHR1 leads to growth plate fusion, preceded by a decrease in chondrocyte proliferation, and ectopic apoptosis of stem-like chondrocytes in the resting zone, effects not observed upon ablation of $G\alpha_s$ (Chagin et al. 2014). Interestingly, chimeric mice containing PTHR1-deficient cells have ectopic hypertrophic chondrocytes throughout the growth plate similar to chimeric mice containing $G\alpha_s$-deficient cells, but in contrast have an expanded zone of flat cells (Bastepe et al. 2004),

thus again showing that ablation of PTHR1 confers a more profound phenotype than does ablation of $G\alpha_s$.

Ectopic differentiated chondrocytes, observed in the experiments with chimeric mice, suggest that the PTHR1-$G\alpha_s$-PKA signaling pathway needs to be active in resting and flat chondrocytes in order to prevent their hypertrophic differentiation. Difficulties to detect PTHR1 mRNA in these cells might be explained by plausible down-regulation of PTHR1 upon constitutive exposure of round and flat chondrocytes to PTHrP, the phenomenon described for other GPCR (Collins, et al. 1989) and for PTHR1 at high PTH levels (Picton, et al. 2000; Tian, et al. 1994).

Gα q and 11

The role of $G\alpha_{q/11}$ G-proteins in chondrocyte differentiation is less known. To date there are no experiments describing genetic ablation or overexpression of either G_q or G_{11} in chondrocytes. The best understanding of the $G_{q/11}$ role in chondrocyte differentiation comes from mice with PTHR1 mutated in the way that it signals via $G\alpha_s$ but not via $G_{q/11}$ (DSEL point mutation, (Guo, et al. 2002)). Developmental delay in chondrocyte mineralization was observed in these mice (Guo et al. 2002), suggesting a function of $G_{q/11}$ (activated by PTHR1) opposed to that of $G\alpha_s$. This role of $G_{q/11}$ signaling is supported by *in vitro* experiments showing that pharmacological activation of PKC, the main downstream signaling pathway of $G_{q/11}$, promotes chondrocyte differentiation (Matta and Mobasheri 2014; Nurminsky, et al. 2007). On the other hand, synergism between PKA and PKC was also reported: (i) inhibition of chondrocyte differentiation by prostaglandin PGE2 requires activation of both PKA and PKC (Li, et al. 2004b); (ii) PTHrP-dependent inhibition of the transcription factor Runx2 requires activation of both PKA and PKC (Li, et al. 2004a).

It is important to note that the effect of delayed mineralization is observed during development of DSEL mice, whereas post-natal growth is rather normal (Chagin et al. 2014; Guo et al. 2002). Furthermore, despite opposing $G\alpha_s$ at mineralization, activation of $G_{q/11}$ by PTHR1 is needed for survival of stem-like cells of the resting zone in the absence of $G\alpha_s$. Activation of PTHR1 is required for round cells to stay quiescent, which is mediated predominantly via $G\alpha_s$ signaling and its absence triggers proliferation of these cells whereas absence of both $G\alpha_s$ and $G_{q/11}$ pathways triggers their apoptosis (Figure 2, (Chagin et al. 2014)). Very little is known about the regulation of this cell population and, accordingly, it is plausible that transition from quiescent round-to-flat chondrocytes is regulated differently as compared to flat-to-hypertrophic transition.

Despite revealing the role of $G_{q/11}$, activated by PTHR1, and providing valuable information of what can be expected from $G_{q/11}$ activation/inactivation, the above experiments do not entirely reveal the role of this G-protein family. Indeed, other potential GPCR can mediate their responses via $G_{q/11}$ signaling in chondrocytes. To date, genetic ablation of $G_{q/11}$ has not been performed and, accordingly, it cannot be excluded that activation of $G_{q/11}$ by other GPCRs impacts chondrocyte differentiation.

Gα12/Gα13

$G\alpha_{12}$ and $G\alpha_{13}$ mainly signal via their downstream target, RhoA (Worzfeld, et al. 2008). Chondrocyte-specific ablation of Rac1, a member of the Rho family of small G proteins, leads to severe growth retardation associated with growth plate disorganization, hypocellularity and decreased proliferation (Wang, et al. 2007). In the ATDC5 chondrocytic cell line, RhoA stimulates cell proliferation and inhibits hypertrophic differentiation (Wang, et al. 2004), which likely occurs via RhoA-dependent actin polymerization, which has been shown to activate Sox9 in chondrocytes (Kumar and Lassar 2009). Furthermore, PTHR1 activates $G\alpha_{12/13}$ family of G-proteins in the osteoblastic cell line UMR-106 (Singh, et al. 2005).

Taken together these observations suggest that $G\alpha_{12/13}$ might be involved in chondrocyte differentiation. We have ablated both $G\alpha_{12}$ and $G\alpha_{13}$ in chondrocytes (using a global $G\alpha_{12}$ KO combined with a Collagen 2-Cre mediated ablation of floxed $G\alpha_{13}$ in chondrocytes), but found no changes either in post-natal bone growth or growth plate morphology (Chagin et al. 2014). This observation suggests that if existing, the role of $G\alpha_{12/13}$ family in growth plate chondrocytes is rather minor.

$G\alpha_i$ in chondrocytes

Proper genetic analysis of G_i function in chondrocytes has never been performed. In general, G_i inhibits the cAMP pathway and opposes $G\alpha_s$ action (Neves et al. 2002). Thus, it can be predicted that G_i would accelerate chondrocyte differentiation by inhibiting the cAMP pathway. In the growth plate NPR-C receptor for c-type natriuretic peptide is expressed by hypertrophic chondrocytes (Yamashita, et al. 2000) and can activate G_i in some settings (Rose and Giles 2008). In articular chondrocytes, G_i can be activated by chemokine receptors, which are expressed by chondrocytes (Borzi, et al. 2000).

Conclusion

Among all G-proteins and GPCRs expressed by chondrocytes, $G\alpha_s$ has the most important role and prevents premature chondrocyte differentiation, whereas PTHR1 is the major activator of $G\alpha_s$ in chondrocytes and ablation of either one leads to abrupt chondrocyte differentiation, premature fusion of the growth plate postnatally and ultimately short stature. The role of $G\alpha_{q/11}$ G-proteins is less defined and $G\alpha_{q/11}$ might have an important role in preventing apoptosis in the absence of $G\alpha_s$ and oppose its action to delay differentiation in the presence of $G\alpha_s$. $G\alpha_{12/13}$ G-proteins seem have no major role in chondrocyte differentiation and bone elongation.

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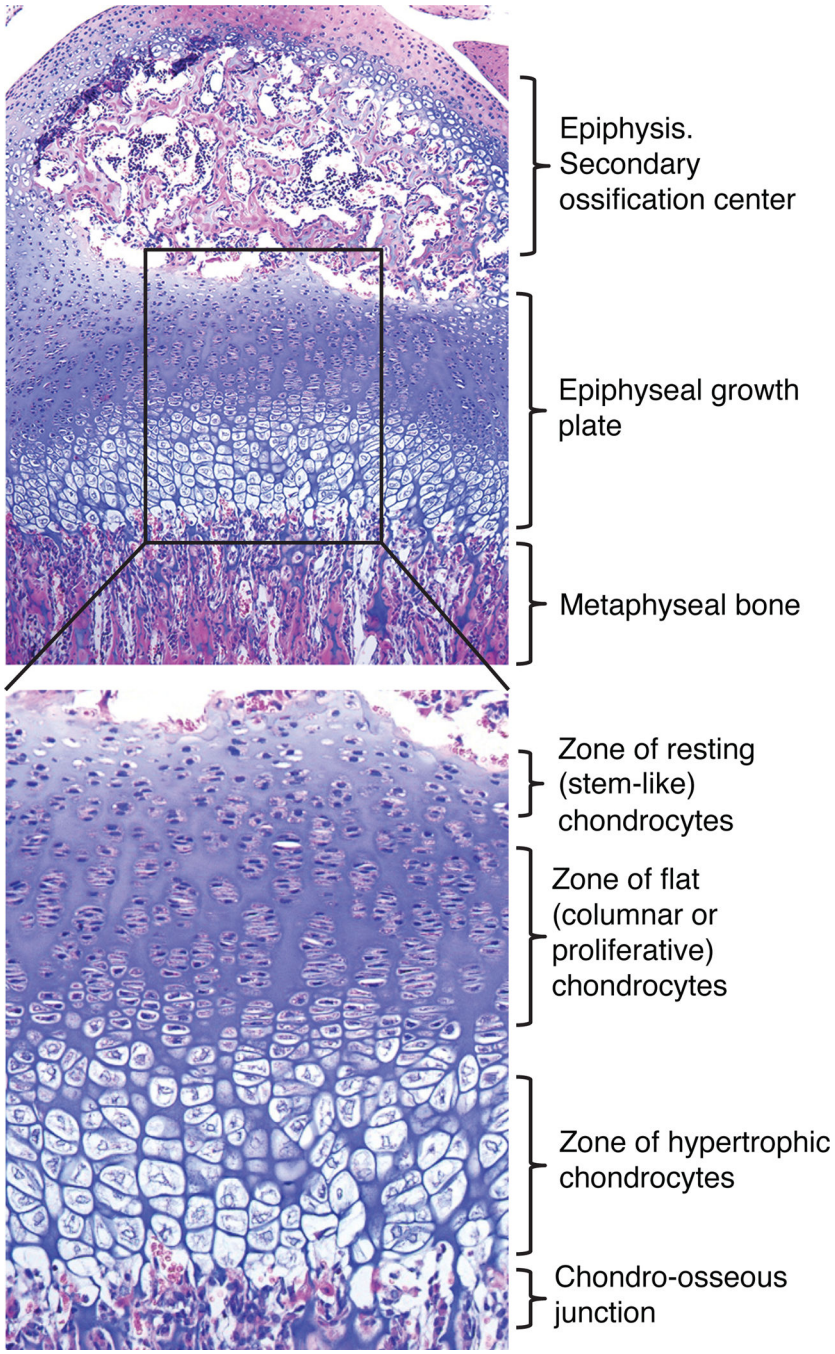

Figure 1.
Structure of the growth plate. Tibial growth plate from 15-days-old mouse is shown. Section is stained with hematoxylin and eosin.

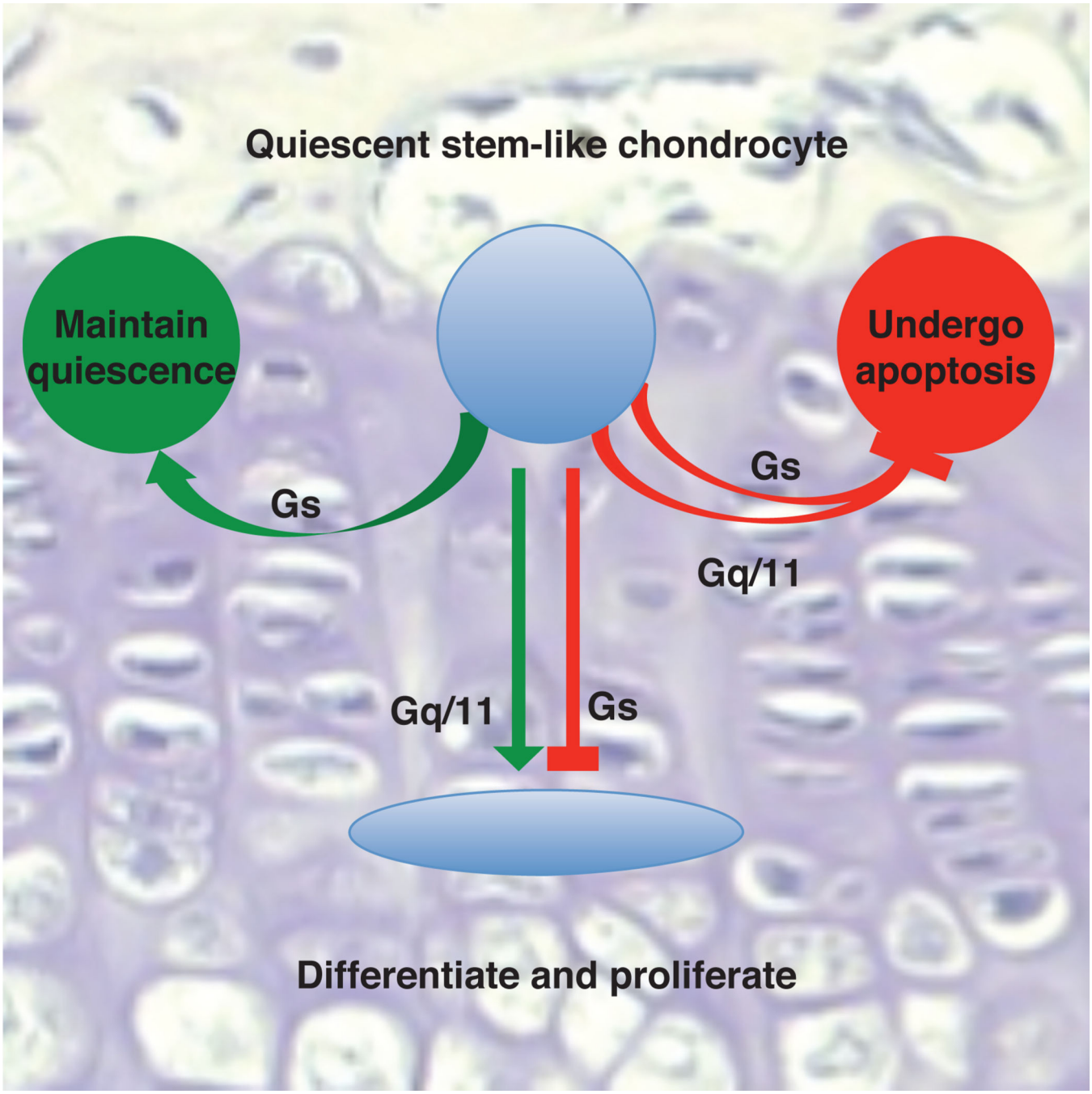

Figure 2.

Receptor for parathyroid hormone maintains quiescence of stem-like chondrocytes via $G\alpha_s$. In the absence of $G\alpha_s$ stem-like chondrocytes are recruited into proliferation whereas without both $G\alpha_s$ and $G\alpha_{q/11}$ signaling these cells undergo apoptosis.